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# Evaluation of polymer choice on immunogenicity of chitosan coated PLGA NPs with surface-adsorbed pneumococcal protein antigen PspA4Pro

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**Abstract**

Polymeric nanoparticles (NPs) are recognized as potential delivery vehicles for vaccines. PLGA is a biocompatible polymer synonymous with polymeric NPs, which can be coated with other polymers such as chitosan that has intrinsic adjuvant properties as well as mucoadhesive properties. Numerous modifications and variations exist for PLGA and chitosan, which can influence the NP characteristics and the resulting immunogenicity. The current study investigated variations for making chitosan coated PLGA NPs incorporating recombinant pneumococcal surface protein A from family 2, clade 4 (PspA4Pro) antigen as a vaccine targeting the vast majority of pneumococcal strains and determine the effect of the polymers on particle size, surface charge, and surface marker upregulation on a dendritic cell (DC) line *in vitro*. PLGA variations tested with the ester-terminal group had the greatest detriment for prospective vaccine use, due to the lowest PspA4Pro adsorption and induction of CD40 and CD86 cell surface markers on DCs. The negatively charged chitosans exhibited the lowest surface marker expressions, similar to the uncoated NP, supporting the commonly accepted notion that positive surface charge augments immunogenic effects of the NPs. However, the study indicated that NPs made from PLGA with an acid terminated group, and chitosan HCl salt, exhibit particle characteristics, antigen adsorption efficiency and immunogenicity, which could be most suitable as a vaccine formulation.

**Keywords**

Nanoparticles, immunogenicity, immunostimulation, vaccine, PLGA, chitosan

## 1. Introduction

The emergence of novel pathogens and rise in antimicrobial resistance has been a key driver of vaccine development in recent times. Among this development, subunit vaccines, using recombinant proteins based on a specific portion of the pathogen, have been gaining interest due to the precision of the resulting immune response and reduced potential for adverse effects [1]. Although recombinant protein antigens exhibit greater safety and precision, one of the main limitations of such antigens is the weak intrinsic immunogenicity, which necessitates additional forms of immunostimulation to induce a sufficient immune response that leads to protection.

The immunogenicity of nanoparticles (NPs) can arise from different avenues, including targeting, uptake and proficient delivery of antigen and adjuvant [2-4]. Poly(lactic-co-glycolic acid) (PLGA) is a particularly popular biocompatible copolymer used to make NPs, and it is already used in various FDA approved therapeutics. The PLGA polymer is available in numerous forms based on the molecular weight, terminal end group and ratio between the lactic and glycolic acid monomers. These parameters may influence NP characteristics and thus interaction with the immune cells or the environment. Administration of an antigen with the NPs have been shown to result in induction of adaptive immune responses and subsequent increases in antigen-specific antibody levels, which leads to protective immunity [4].

Adsorption of the antigen onto the NP surface, through electrostatic and ionic interaction, is a simple approach for the incorporation of the antigen to the NP [5]. The presence of the antigen at the particle surface also mimics pathogens which have the antigen in the cell wall, such as the pneumococcal surface protein A (PspA) in *Streptococcus pneumoniae*. This antigen is of interest as it potentially offers protection against a wide range of pneumococcal strains, compared to the currently available conjugate vaccine [6]. Furthermore, adsorption avoids potential degradation and denaturation of the antigen structure during preparation of the NPs, which generally involves contact with organic solvents and high energy homogenisation [7].

Incorporation of chitosan on the surface of polymeric NPs has been shown to improve immunogenicity and delivery of encapsulated material at mucosal sites [8]. The interest for use in vaccine applications is commonly attributed to several mechanisms: the immunogenicity from the positive charge of the amine group, increase in residence time and antigen presentation through mucoadhesion, and the reversible opening of tight gap junctions between epithelial cells [9]. Another mechanism occurs through recognition by the Toll-Like Receptor (TLR) pathway and the induction of innate immune responses, generating type I interferons that mediate maturation of dendritic cells (DCs). In addition, chitosan can induce mitochondrial stress and the subsequent increase in reactive oxygen species (ROS) production [9, 10]. This results in the opening of mitochondrial permeability transition pores and release of mitochondrial DNA, which is likely recognized as a danger-associated molecular pattern (DAMP) that serves as a trigger for cyclic GMP–AMP (cGAS) activation. Furthermore, chitosan has the potential to be modified, with studies on various forms of chitosan claiming improvements in solubility, mucoadhesiveness, and penetration enhancement that can contribute to the delivery and immunogenicity of vaccine formulations [9, 11].

DCs are arguably the most crucial of potent antigen presenting cells (APCs), which exhibit highly phagocytic capacity and can initiate adaptive immune responses leading to memory and protection. Substantial evidence indicates that interactions depend on the physicochemical properties, especially the surface chemistry of NPs, which influences the recognition and uptake [12, 13]. Particle size also influences uptake by DCs, with a size of 300 nm exhibiting high uptake efficiency; although there are still ambiguities in how size relates to immunogenicity [13, 14]. The stimulation of DCs by NPs

ultimately leads to metabolic changes and upregulation of costimulatory molecules, such as such as CD80, CD86, and CD40, and production of cytokines. All which serve to stimulate immature T cells during antigen presentation [13]. During the development of an adaptive immune response, the function of DCs is immensely influential in initiating differentiation for tolerance, memory, and T-helper 1 (Th1), Th2 and Th17 responses.

The aforementioned properties of NPs such as those arising from the use of different PLGAs and coating with chitosans of various properties, are highly important for driving the desired immune response outcome. By understanding how such differences in the polymers and how it affects the subsequent particle properties and the immunogenicity on the DCs, the optimal combination could be chosen for the desired application. This study investigated the effect of different PLGA polymers and chitosans on the resulting characteristics of chitosan coated PLGA NPs, and the subsequent immunogenicity of the NPs on DCs through upregulation of cell surface activation markers in an *in vitro* JAWSII cell model.

## 2. Materials and methods

### *2.1. Materials*

PLGA (Lactide:glycolide (50:50), acid terminated, average Mw 7-17 kDa), (Lactide:glycolide (50:50), ester terminated, average Mw 7-17 kDa), (Lactide:glycolide (50:50), acid terminated, average Mw 24-38 kDa) and (Lactide:glycolide (75:25), acid terminated, average Mw 4-15 kDa) polyvinyl alcohol (PVA), were obtained from Sigma, UK. Chitosan hydrochloride (HCl), carboxymethyl chitosan (CMC), chitosan oligomer, chitosan glutamate, were obtained from Heppe Medical Chitosan GmbH, Germany. The dendritic cell line, JAWS II (CRL-11904TM), was purchased from American type culture collection (ATCC). Alpha minimum essential medium (α-MEM), fetal calf serum (FCS), trypLE, sodium glutamate, and granulocyte macrophage colony-stimulating factor (GM-CSF) were purchased from Life technologies, UK. Purified water from a Millipore Purification System (Billerica, MA, USA) was used throughout the study.

### *2.2. Preparation of NPs*

PLGA NPs were prepared using the solvent evaporation method. Briefly, 200 mg of the PLGA polymer (Table 1) was dissolved in 2 mL dichloromethane (DCM), added dropwise to 3 mL of 10 % w/v PVA while ultrasonicating (Q125 Ultrasonic Homogenizer, Qsonica, USA) for 2 min at 65% amplitude on ice. The resulting O/W emulsion was added dropwise to 4 mL of 0.5 % PVA and stirred for 3 h to allow the DCM to evaporate. The resulting suspension was centrifuged at 80,000 ×g in a Beckman Coulter type 70.1 rotor for 30 min, the supernatant was discarded, and the pellet was resuspended in 3 mL of water, with 30 sec agitation in a bath sonicator (Sonorex, Bandelin, Germany).

### *2.3. Coating with chitosan*

The PLGA NPs were coated with the chitosans (Table 2) through electrostatic interactions. The chitosans were first dissolved in 1% acetic acid and added to the second aqueous phase during PLGA NP preparation, at a final concentration of 1 mg/mL or as indicated in the experiment. The NPs were processed in the same manner as without chitosan.

### *2.4. Surface adsorption with PspA4Pro and quantification using HPLC*

To surface adsorb NP +/- chitosan with PspA4Pro, 1 mL of 1 mg/mL PspA4Pro was added to the 3 mL of 25 mg NP suspension (produced in 2.2 and 2.3) and mixed with a magnetic stirrer at room

temperature for 1 h. The NP suspension was ultracentrifuged at 80,000 ×g in a Beckman Coulter type 70.1 rotor. The adsorption of PspA4Pro on the NPs was quantified using a previously developed high performance liquid chromatography (HPLC) method in our group [15]. The adsorption was calculated by the difference between the initial amount of PspA4Pro and the amount detected in the supernatant

*2.5. Particle size and zeta potential characterisation*

The size of particles and polydispersity index (PDI) was measured at 20°C, in deionised water, at a concentration of 0.1 mg/mL, using dynamic light scattering (Zetasizer Nano, Malvern Instruments Ltd, Malvern, Worcestershire, UK). The zeta potential was measured by electrophoretic mobility using a capillary cell in the same machine. The hydrodynamic diameter measurements are reported as intensity

*2.6. Quantification of coated chitosan*

The quantity of chitosan adsorbed to the surface of NPs was determined by the fluorescamine assay. Chitosan coated NPs were ultracentrifuged at 80,000 ×g and the supernatant was obtained. A 50 µL aliquot of fluorescamine in DMSO (2 % w/v) was added to 20 µL of the supernatant sample in wells of a 96-well plate. The reaction continued for 3 h in the dark and the fluorescence in the wells were measured at 390 nm excitation and 515 nm emission, using a microplate reader (CLARIOstar® BMG Labtech, Germany). A standard curve was prepared for each type of chitosan by spiking blank supernatant with predefined quantities of the chitosans. The adsorption was calculated by the difference between the initial amount of chitosan and the amount detected in the supernatant

*2.7. Evaluation of NP immunogenicity and toxicity in JAWSII cells*

JAWSII cells were seeded at a concentration of 50,000 cells/mL in 24 well plates, with complete medium (α-MEM containing ribonucleosides, deoxyribonucleosides, 4 mM L-glutamine, 1 mM sodium pyruvate supplemented with 20% FCS, 5 ng/ml murine GM-CSF and 1% antibiotic/antimycotic solution. The cells were incubated at 37 °C, 5% $CO_2$ for 48 h, the NP formulations were added to the wells at a final concentration of 0.1 mg/mL for PLGA-only experiments (Section 3.2) and 0.05 mg/mL for chitosan coated NP experiments (Sections 3.4-3.7), and the cells were incubated for a further 24 h. The plate was centrifuged at 300 ×g for 5 min, the supernatant removed, 100 µL of TrypLE added to each well, and incubated for 5 min. The cells were removed from the wells with 1 mL of PBS, transferred to microtubes, centrifuging at 300 ×g for 5 min then removing the supernatant. The cells were washed by resuspending the cells in PBS and repeating the centrifugation. The cells were stained with anti-mouse CD40-FITC and CD86-PE, washed and resuspended in 7AAD (1:500). The cells were analysed using a BD Accuri™ C6 flow cytometer, and gated according to the signals (Figure S1). The toxicity was determined by gating for the 7AAD negative cell population and expressed as the live proportion within the total population of cells. The immunogenicity was determined by the CD40 and CD86 surface expressions, which were measured as the mean fluorescence intensity (MFI).

*2.8. Statistical analysis*

Results are expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with Tukey's pairwise comparison or unpaired t-tests were used to compare groups using Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was determined as $p<0.05$.

## 3. Results

*3.1. Characterisation of PLGA NPs*

NPs prepared from PLGA with differences in the glycolic acid:lactic acid ratio (50:50 and 72:25), polymer size (7-17 kDa and 24-38 kDa), and polymer terminal group (ester and carboxylic acid), produced particles of similar sizes at ~150 nm and PDI <0.2 and zeta potential of ≤-14 mV (Figure 1A-C). There were no statistically significant differences between the characteristics resulting from different PLGA polymers. After surface adsorption of PspA4Pro, the NP size did not significantly increase. This is despite the high degree of PspA4Pro adsorption to the particle surfaces at >90% for REF, HMW and LAC (Figure 1D). EST was alone in exhibiting significantly lower PspA4Pro adsorption of 16 % (Figure 1D). The PDI increased after protein surface adsorption but the NPs remained relatively homogenous with all PDI values under 0.2. The zeta potentials for all polymer particles were all negative in the range of -14 to -30 mV before coating with PspA4Pro. After coating, the zeta potentials were more negatively charged, with EST gaining additional negative charge of ~10 mV.

*3.2. Immunogenicity of PLGA NPs*

The degree of immunogenicity of the PLGA NPs were evaluated by incubating the formulations with the immortalised DC cells (JAWSII) and measuring surface marker expression of CD40 and CD86, indicative of DC activation. To initially determine the optimal NP concentration and incubation time for measuring the response, REF NPs at concentrations from 0.1 to 1000 μg/mL, were incubated with JAWSII cells for 24 h (Figure S2A&B). The REF NPs at 100 μg/mL were also incubated for a 48 h time course (2, 4, 16, 24, 48 h) (Figure S2C&D). The NP stimulated the CD40 and CD86 JAWSII expression in a concentration dependent manner, with 100 μg/mL inducing significantly higher expression compared to culture media alone. Over the 48 h time course, the NPs exhibited positive relationship, showing increased surface marker expression with increasing incubation time.

The LAC exhibited significantly lower degree of cell viability compared to the other PLGAs, although this was still above 85% viability (Figure 2A). With the exception of the EST, the NPs with and without the adsorbed PspA4Pro, were shown to induce the upregulation of the surface markers (Figure 2B&C). As the particle sizes were similar between the different polymers, the relatively lower magnitude of negative surface charge and greater hydrophobicity of the ester PLGA NP appears to have significant influence on the NP to DC interaction.

The toxicity and immunogenicity were unaffected by the presence of PspA4Pro, indicating the antigen itself has no role in the upregulation of surface markers in DCs in this assay. This was supported by the lack of CD40 and CD86 upregulation by PspA4Pro alone (Figure S3). The LAC was the most significant contributor to immunogenicity (Figure 2B&C), likely due to the presence of a negative charge, which is absent in the EST. The HMW (24-38 kDa) also induced lower surface marker upregulation relative to the REF (7-17 kDa).

*3.3. Coating with chitosan*

Chitosans of varying molecular configurations (Table 2) were investigated for particle characteristics and immunogenicity, using REF as the PLGA NP component. After coating NPs with different chitosans, the resulting NP exhibited little changes in particle size, apart from CMC, which exhibited an average diameter of over 200 nm compared to the uncoated NP with 180 nm (Figure 3A). The PDI values were mostly under 0.3, with the exception of the PspA4Pro adsorbed Glycol chitosan coated NP (Figure 3B). The zeta potential of the various chitosan coated NPs varied from +30 mV of the glycol chitosan to -30 mV of the CMC (Figure 3C). These differences reflect the differences in the ionisation states of the polymers.

The addition of PspA4Pro generally increased particle sizes, with the glycol chitosan exhibiting an increase that resulted in the largest particles outright at 600 nm. This increase in size was not entirely

correlated to the amount of PspA4Pro adsorption, as HCl, glutamate and glycol chitosans exhibited near 100% PspA4Pro adsorption, but only the glycol resulted in such significant increase (Figure 3A). Furthermore, the zeta potentials decreased to be closer to neutral for HCl, LMW, and TMC chitosans, but increased for glutamate and glycol chitosans (Figure 3C).

The PspA4Pro adsorption efficiency also varied greatly with different chitosans. PspA4Pro adsorption and zeta potential appeared to exhibit some correlation, as the negatively charged CMC and oligomer chitosan coated NPs exhibited under 10% adsorption efficiency, whereas the positively charged HCl, glutamate and glycol chitosan coated NPs exhibited over 90% efficiency (Figure 3E).

Measurement of chitosan adsorption revealed that the degree of adsorption varied between each chitosan (Figure 3D), when a constant chitosan concentration was used during the preparation process (1 mg/mL). The HCl, TMC, CMC and glycol chitosans exhibited the highest adsorption onto particles at 55-70 %, while the LMW, oligomer and glutamate chitosans were below 45 %. The chitosan adsorption was also not correlated to surface charge as CMC, which has negative zeta potential, exhibited a high degree of chitosan surface adsorption.

*3.4. Chitosan immunogenicity related to adsorption efficiency*

Incubation of the chitosan coated NPs with JAWSII cells resulted in various degrees of CD40 and CD86 expression (Figure 3F&G), with the chitosan HCl exhibiting significant increases in expression compared to non-chitosan coated NPs. There were minimal differences between CMC, oligomer chitosans compared to uncoated NP for CD40. The high adsorbance of CMC, despite the low CD40 and CD86 upregulation, indicates that CMC has low intrinsic immunogenicity. This effect by CMC and oligomer could indicate that the zeta potential could have the greatest influence on CD40 and CD86 upregulation in this cell model. The effects of the chitosans did not appear to be correlated solely to any other specific NP characteristic. However, the zeta potential in the positive range did not correlate to the degree of surface marker upregulation. This could be observed for glycol chitosan exhibiting a high positive zeta potential, but inducing less upregulation compared to HCl, which had a lower zeta potential.

*3.5. Effect of chitosan preparatory concentration*

As the chitosan loading differed for each chitosan, the correlation between preparatory chitosan concentration, the resulting chitosan adsorption efficiency, and the degree of DC activation with equivalent amounts of chitosan, needed to be elucidated. The relationship between the chitosan concentrations in the emulsion during NP preparation and the final adsorption onto the NPs was determined by preparing NPs coated with the positively charged chitosans at different concentrations (0.2, 0.5, 1.0 and 2.0 mg/mL).

The change in particle size, resulting from the coating, exhibited differing patterns between different chitosans (Figure 4). The size difference between the lowest and highest tested preparatory concentrations (0.2 and 2 mg/mL) ranged from no change for HCl and TMC, to an increase of ~120 nm for LMW and Glycol. The PDI was similar between chitosan preparatory concentrations, indicating consistent increases in homogenous particle size. The zeta potential exhibited a concentration dependent pattern, with the largest change occurring between the 0.2 and 0.5 mg/mL preparatory concentrations. HCl, TMC and Glycol possess zeta potentials close to neutral at 0.2 mg/mL, indicating that the PLGA still has a large influence on the charge characteristics of the resulting NPs.

The loading capacity profile is also unique between different chitosans. HCl exhibits increasing loading capacity with increasing preparatory concentration, whereas glycol chitosan had very little change

above the lowest concentration. This is further shown in in the adsorption efficiency (Figure 4D) which shows HCl with a relatively constant adsorption efficiency 50-60%, whereas the glycol exhibits a decrease in adsorption efficiency with increasing preparatory concentration. When comparing the loading capacity to the particle characteristics (Figure 4E), the results for LMW and Glycol chitosan are contrasting. The size, zeta potential and loading capacity appear to be correlated for the LMW, whereas the plateau observed for the Glycol appears to correlate with the zeta potential, but not with the continued increase in particle size (Figure 4A).Overall, the chitosan HCl appeared to have the highest adsorption efficiency and thus the highest amount of chitosan coating at any starting chitosan concentration. A notable anomaly for the total adsorbed chitosan was the glycol chitosan, which exhibited a plateau in the adsorbed amount (Figure 4E) even when the preparation concentration increased.

The varying amounts of adsorbed chitosan were tested in the JAWSII cells, which indicated a mixed response between the total amount of adsorbed chitosan and the expression of surface markers (Figures 5). LMW chitosan was the only chitosan, with which the highest chitosan concentration and chitosan loading capacity, resulted in the highest surface marker expression. The correlation could also be applied to glycol, which had plateaus after 0.2 mg/mL for both loading capacity and surface marker upregulation. The other chitosans of HCl and glutamate, exhibited some degree of concentration dependent surface marker upregulation, but the response did not follow chitosan concentration. This was especially apparent for HCl, which exhibited a plateau in both CD40 and CD86 expressions, despite the increase in the chitosan loading capacity. This could be an indication that there is an upper limit of DC stimulation for chitosan HCl, which is limited through surface charge, as the particles with zeta potential above +20 mV exhibited similar CD40 and CD86 upregulation.

### *3.6. NP characteristics with equal chitosan loading capacity*

As the chitosan loading capacity was shown to be influential in particle characteristics and immunogenicity, chitosan coated NPs with similar loading capacities were prepared in order to compare the effects of the chitosans at comparable loading. The adsorbed amount, corresponding to 5% loading capacity, was chosen and prepared using the appropriate chitosan concentration during preparation for each chitosan. The resulting particles exhibited predictable particle properties when produced with equal chitosan loading capacities. As seen in Figure 6A, the LMW, Glutamate and Glycol coatings resulted in the largest increases in particle size, whereas the HCl remained similar to the uncoated chitosan. The zeta potential ranged from +20 mV to +40 mV (Figure 6C), with LMW having the highest compared to the rest.

### *3.7. Immunogenicity with equal chitosan loading capacity*

The chitosan HCl consistently exhibited the highest expression of both CD40 and CD86 compared to the uncoated NP (Figure 6D&E), which suggests that the HCl chitosan is the most immunogenic when the quantity is kept constant. The HCl has been shown to exhibit not only high adsorption efficiency, but also high PspA adsorbance and high immunogenicity. In contrast, the glycol exhibited the lowest surface marker expressions. When PspA4Pro was adsorbed to the particles, the pattern of the results were similar, with chitosan HCl still exhibiting high surface marker expression (data not shown), suggesting that PspA4Pro on the particle surface does not contribute to immunogenicity of the particles. The toxicity to the cells were minimal (Figure 6F), indicating that the immunogenic effects and processes indicated by the upregulation in CD40 and CD86 were not detrimental to the cells.

## 4. Discussion

PLGA and chitosan have been of interest as materials for delivery vehicles, due to biocompatibility, efficacy and accessibility. The potential application of the combination of PLGA and chitosan for vaccines, was confirmed in this study, as PLGA and chitosan polymers of different characteristics were evaluated for NP characteristics and immunogenicity.

### *4.1. Effect and differences between PLGA variations*

The Initial characterisation of the PLGA NPs suggested that the polymers did not contribute to any significant difference in particle size or surface charge. However, the EST PLGA NPs exhibited significantly lower PspA4Pro adsorption compared to the other PLGAs (Figure 1). The difference in adsorption could be due to the greater surface hydrophobicity stemming from the ester end-group of the polymer. This result has been observed in other studies and appears to be equally influenced by the molecular properties of the protein [16].

In these studies, the expression of CD40 and CD86 cell surface markers, were used to determine immunogenicity, as they are important markers for DC activation. CD40 ligation is known to be an important process for maturation of DCs and contributes to subsequent activation of T cells through mechanisms such as the secretion of IL-12 [17]. The CD40 upregulation is also associated with CD86 and MHC II upregulation, which are important for co-stimulation and antigen presentation, respectively [18].

Despite the lack of major differences in particle characteristics made from different PLGAs, the immunogenicity was consistently lower for the EST PLGA NPs. The acid terminated PLGAs with lower molecular weight exhibited the highest immunogenicity, suggesting that the end groups have a significant contribution to the interaction with DCs, without affecting characteristics such as size and surface charge. Surface characteristics like hydrophobicity is widely recognised to influence particle interaction with DCs, however the greater hydrophobic nature of esters would be predicted to increase cell uptake [19] and immunogenicity [20], which is contrary to the results here. It could be that some ester end groups undergo hydrolysis to form acid groups and thus do not exhibit the immunogenicity associated with hydrophobicity. The HMW PLGA NP also exhibited significantly lower induction of CD40 and CD86 compared to the REF and LAC PLGAs. A potential explanation could again be due to the lower number of end terminal groups in the HMW compared to the REF, for a given equal mass. This would also reinforce the importance of end terminal group as demonstrated by the results from the ester end terminal group.

### *4.2. Effect and differences between chitosan variations*

Chitosan is a widely studied and recognised component of experimental vaccine formulations. However, as shown in the results, there are several variants which can exhibit significant differences in the particle size and surface charge, as well as surface adsorption of PspA4Pro. To determine the effects of each chitosan, the initial goal was to determine the resulting characteristics of NPs made from using constant coating concentrations during preparation (Section 3.3). It was apparent that each chitosan resulted in unique particle characteristics. An expected confirmation was that the negatively charged chitosans such as CMC and oligomer would not be appropriate for antigen adsorption. This was due to the negative charge resulting in reduced affinity for protein molecules which may be negatively charged at physiological pH. The chitosans were shown to contribute not only to the adsorption efficiency of PspA4Pro, but also to the particle characteristics, as seen with the increased average particle size after coating with PspA4Pro (Figure 3A). The zeta potential also changed after PspA4Pro coating, but there were no patterns associated with the change. HCl, LMW and TMC

chitosans decreased in zeta potential, whereas it increased with the glutamate and glycol chitosans. Furthermore, the low PspA4Pro adsorption on the CMC and oligomer NPs (Figure 3E) could be attributed to the negative zeta potential. However, uncoated PLGA NPs exhibited high adsorption (Figure 1D). This suggests that the differences in PspA4Pro adsorption may not be solely dependent on the zeta potential, but also other molecular interactions such as hydrophobic interactions between PspA4Pro and the NP surface [21].

To examine the effects of chitosan concentration on the particle characteristics, the effect of preparatory concentration of chitosans was investigated. The results were used to prepare particles with constant amounts of adsorbed chitosan. The effects on particle characteristics and immunogenicity from different chitosan concentrations during preparation demonstrated unique patterns between chitosans (Figure 3-6). Chitosan HCl exhibited the properties which may be most ideal as a candidate for vaccine formulation, considering the particle characteristics, adsorption of chitosan and PspA, and the immunogenicity. Evaluating the immunogenic effects of chitosan coating on the NPs revealed that the size, PDI, and zeta potential values alone could not fully account for the differences in CD40 and CD86 cell surface upregulation. As the changes in particle characteristics are unique between each chitosan, the upregulation of the cell surface markers is likely as a result of the changes to both particle characteristics, as well as the intrinsic immunogenic effects of the chitosan polymer itself. For example, chitosan HCl generally appeared to exhibit greater upregulation of both CD40 and CD86 than the LMW and glutamate chitosans, despite the NPs exhibiting similar particle sizes and surface charge. These differences in immunogenicity could be due to differences in how each chitosan variation interacts with the DCs or how it induces immunogenicity, such as through the cyclic GMP–AMP receptor stimulator of interferon genes (cGAS-STING) signalling pathway [10].

In addition to the resulting particle properties, chitosan HCl may be beneficial from a production perspective as it is a salt. This enables solubilisation of chitosan HCl in water, as opposed to the non-salt chitosans. The solubilisation could potentially enable coating of particles without requirement for acidifying the solution during that stage of preparation, and thus reduce potential for degradation of the particle components. Of the chitosan salt form tested, it is also interesting to note that the glutamate salt chitosan exhibited different immunogenic characteristics to the HCl salt. This could be due to the difference in salt group or the difference in the molecular weight range differences between the HCl (30-400 kDa) and glutamate (30-200 kDa).

This leads to the aspects of the chitosans, molecular weight and degree of deacetylation, which could not be fully controlled. The chitosans could be clustered into two size groups; a larger size range (30-500 kDa) for HCl, CMC and Glycol, and a narrower size range (30-200 kDa) for LMW, TMC, Oligomer and glutamate. There is also further potential for differences in polymer size distribution within these size ranges, which many affect the interaction. Although the degree of acetylation for the tested chitosans were all >75%, it is also unclear how much influence the remaining 25% would have for each chitosan. Existing literature indicates that both high and low molecular weight chitosan can exhibit immunogenicity and could be intertwined with the degree of acetylation [22, 23]. Despite these uncertainties, chitosan HCl exhibits not only low particle size change upon coating and high immunogenicity, but also greater versatility during NP preparation due to the enhanced solubility offered by the salt form.

### *4.3. Outlook of the PspA4Pro adsorbed, chitosan coated, PLGA NP as a vaccine against S. pneumoniae*

In addition to NP characteristics and adjuvant properties, an effective vaccine formulation must contain an appropriate antigen which can provide the immune system with a suitable target. In this experiment, PspA4Pro, which is a recombinant protein found on the surface of *S. pneumoniae,* was

incorporated on the surface of the NPs. The protein offers potentially greater protective coverage between different *S. pneumoniae* serotypes, compared to commercially available conjugate pneumococcal vaccines [24]. The immunogenic properties of PspA4Pro are also advantageous compared to polysaccharide antigen that are T cell independent and induce poor memory responses [24]. In the current study, the incorporation of the PspA4Pro to the surface of the particles could be formed through the formation of electrostatic and hydrophobic interaction with the surface of the PLGA NP. This preparation method exhibited high adsorption efficiency (Figure 3E) and could be useful for the delivery to the immune cells of the lung, in combination with the immunogenic properties of the chitosan HCl coated PLGA NPs shown through the upregulation of the CD40 and CD86 on the DCs (Figure 6D&E). Considering the pathology of *S. pneumoniae* infection, inhalation of NPs into the lungs would be a suitable avenue of delivery, as it mimics the natural pathology of the bacteria. Although PspA4Pro is expected to cover most pneumococcal strains, there have been evidence to indicate that PspA4Pro alone is insufficient for coverage of all serotypes. PspA4Pro generated serum IgG with binding to bacteria with expression of PspA from clades 3, 4 and 5 (family 2), but no binding to bacteria expressing PspA from clades 1 and 2 (family 1) [25]. Further investigation is ongoing to incorporate another pneumococcal protein which could resolve this potential issue.

### *4.4. Future direction of in vitro assays in analysing NP impact within the lung environment*

In order to further elucidate translatability of the results to the larger, overall immune response, there are several aspects which could be investigated in the future. These would include investigating the effect of NP uptake by macrophages, which can account for a high degree of leukocytes present in areas such as the lungs, exhibit high phagocytic capacity, and are known to take up particles of a wide range of sizes and forms [26]. They have also been shown to have a role in the modulation of cytokines and T cells, affecting subsequent responses such as mucous production and inflammatory cytokines [27]. In addition, epithelial cells of the mucosal linings are also known to take part in antigen presentation through surface expression of MHC II and subsequent presentation [28], thus it would be beneficial to understand the significance of contribution of these other entities. In terms of the DC response, establishing the correlation of the CD40 and CD86 upregulation to the antigen specific antibody production and T cell response would offer a valuable means for evaluating and optimising formulations in vitro, in an ethical acceptable manner. By optimising the uptake and interaction of the formulation with the cells present in the environment to induce the desired processes, there is potential for even greater protective effects, allowing for lower dosages and thus potentially reduced booster does, as well as cheaper production.

## 5. Conclusion

NPs made from ester-ended PLGA polymers exhibited significantly lower immunogenicity in JAWSII cells compared to acid-ended PLGA NPs, which could correspond to the difference in surface charge. Upon coating the NP with chitosan, NPs coated with negatively charged chitosans generally exhibited lower immunogenicity compared to NPs coated with positively charged chitosan. The adsorbance of chitosan varied and upon coating with consistent loading, the NP coated with chitosan HCl (30-400 kDa Mw), induced high immunogenicity, through CD40 and CD86 expression on JAWSII DCs.

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**Figure Captions**

**Figure 1.** Particle characteristics of NPs prepared from REF, EST, HMW and LAC PLGA polymers. The particle size **(A)**, PDI **(B)**, and zeta potential **(C)** with and without PspA4Pro adsorption. The adsorption efficiency of PspA4Pro to PLGA NPs **(D)**. (n=3 independently prepared batches, mean±SD, significantly higher adsorption efficiency of a group over another is indicated by the respective letter, $p<0.05$).

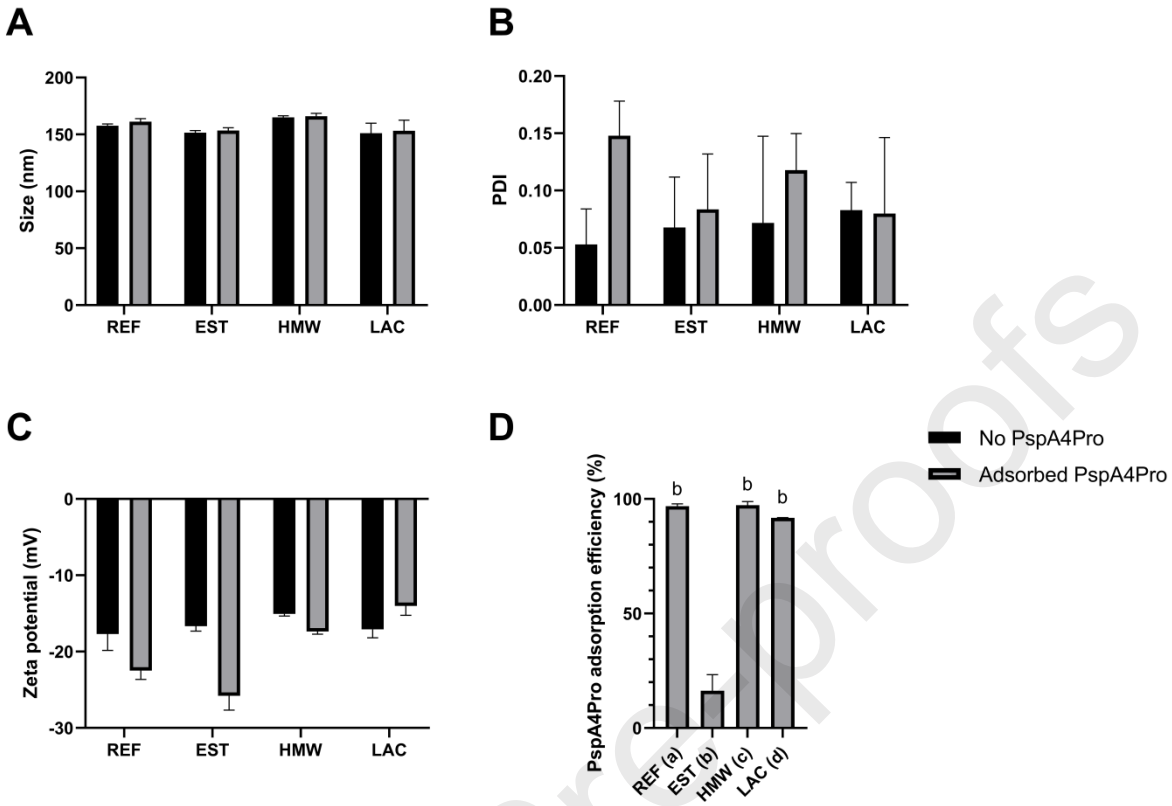
**Figure 2.** Toxicity of PLGA NPs **(A)**, upregulation of CD40 **(B)** and CD86 **(C)** surface markers after incubation for 24 h (n=4, representative of 3 independent experiments with independent batches of NP, mean±SD. Significantly higher surface marker expression between the groups is indicated by the respective letter, $p<0.05$).

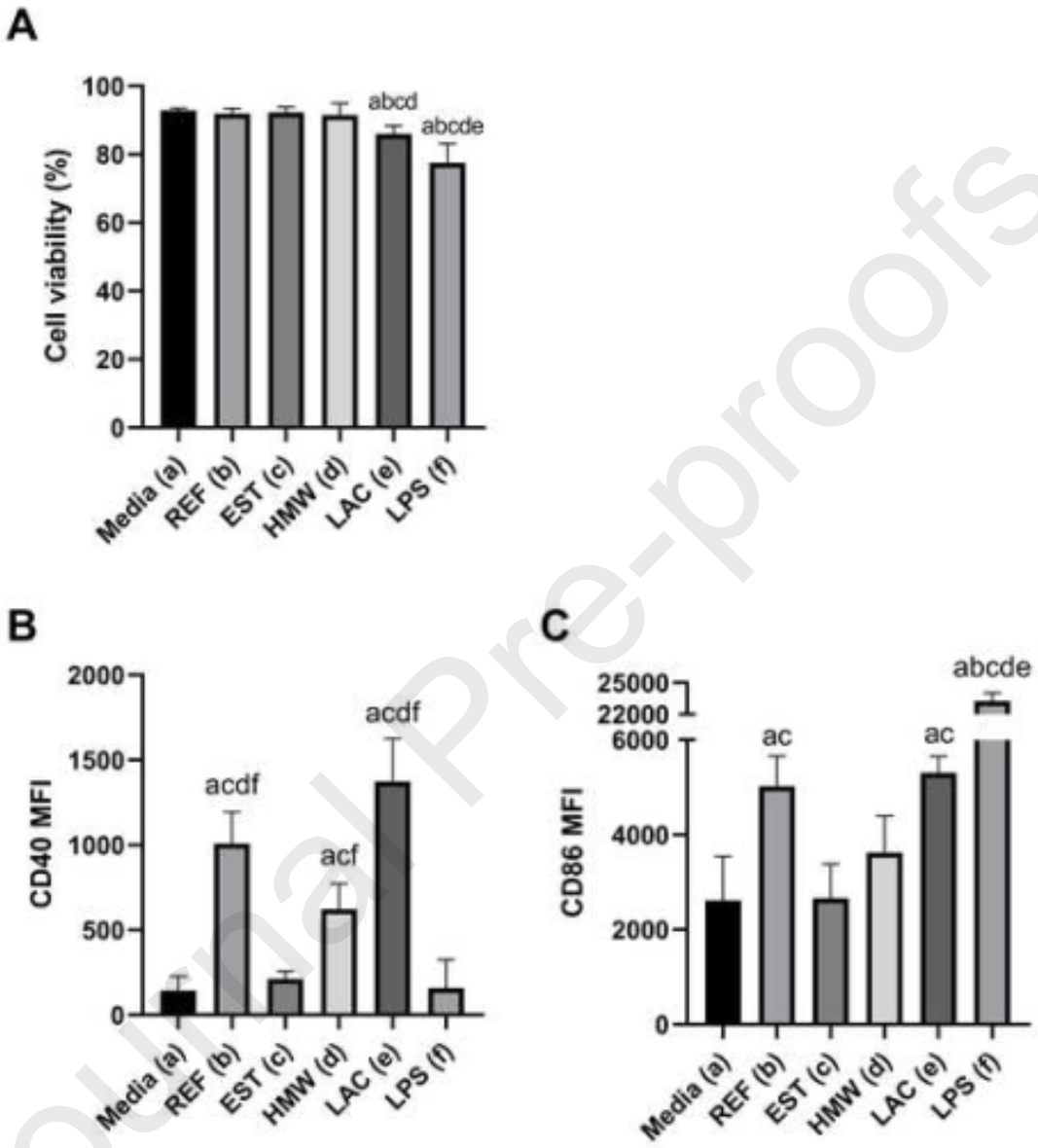
**Figure 3.** The size **(A)**, PDI **(B)** and zeta potential **(C)** of PLGA NP coated with chitosans and PspA4Pro, and the adsorption efficiency of chitosans on PLGA NPs **(D)** and PspA4Pro on chitosan coated PLGA NPs **(E)** (n=3 independent batches, mean±SD). Upregulation of CD40 **(F)** and CD86 **(G)** surface markers after incubation with PLGA NPs for 24 h (n=4, representative of 3 independent experiments with independent batches of NP, mean±SD) (significantly higher difference compared to another group is indicated by the respective letter, $p<0.05$).

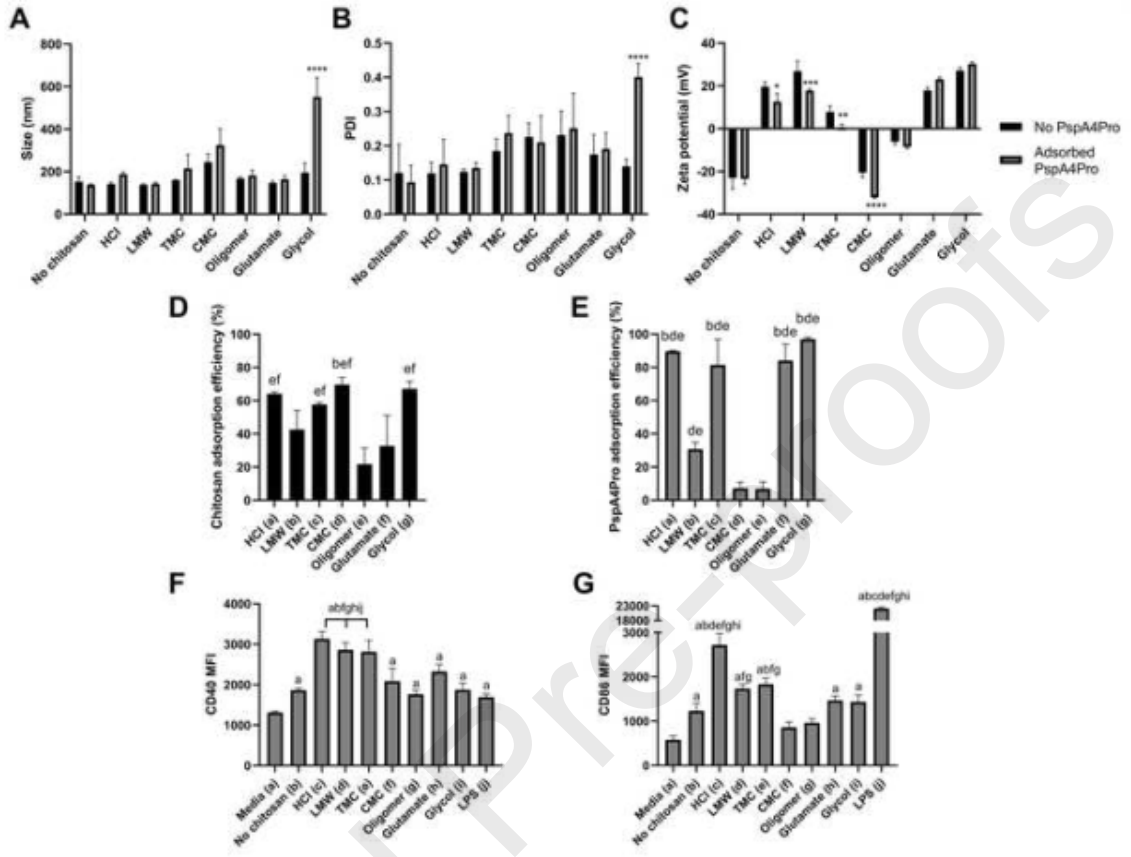
**Figure 4.** The particle size **(A)**, PDI **(B)**, and zeta potential **(C)** of PLGA NPs coated with various preparatory concentrations of chitosans (0.2, 0.5 1 and 2 mg/mL). The loading capacity **(D)** and adsorption efficiency **(E)** of the different chitosan at 0.2, 0.5, 1 and 2 mg/mL chitosan concentrations during preparation (n=3 independent batches, mean±SD, a statistically significant correlation according to linear regression between the preparatory concentration and response is indicated by the * above the group, $p<0.05$).

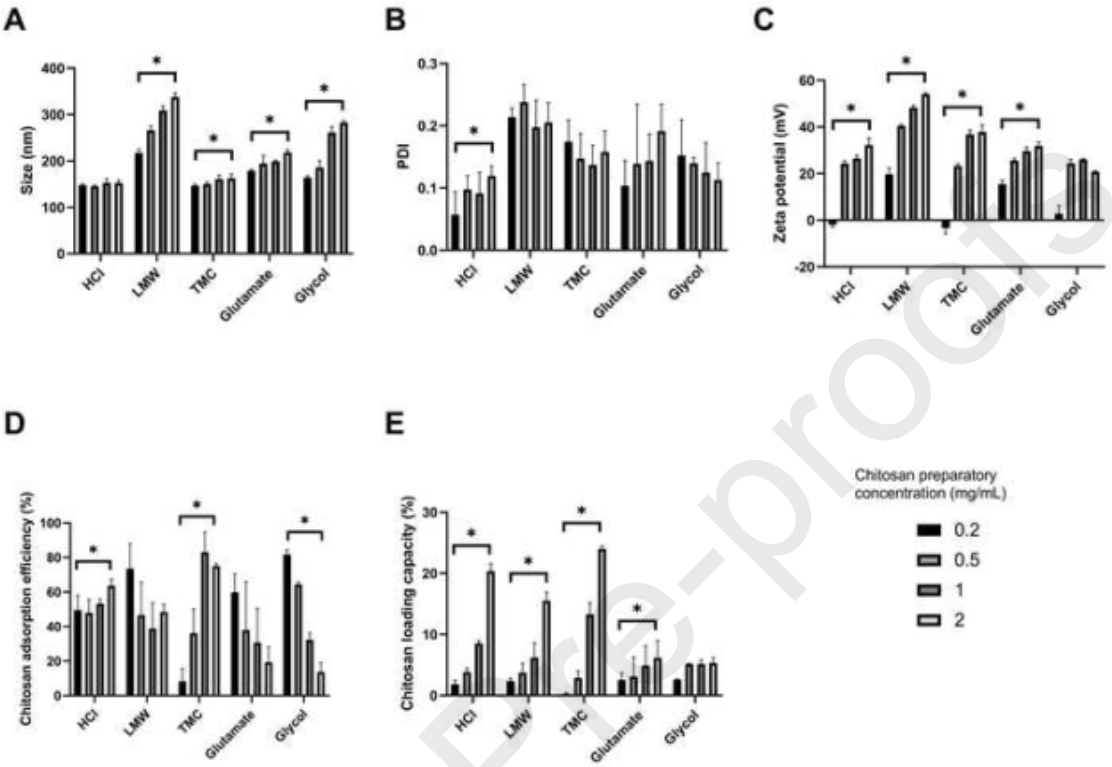
**Figure 5.** CD40 (black) and CD86 (grey) surface upregulation on JAWSII cells after incubation with PLGA NPs coated with varying preparatory chitosan concentrations (0, 0.2, 0.5 1.0 and 2.0 mg/mL) and derivatives, HCl **(A)**, LMW **(B)**, TMC **(C)**, Glutamate **(D)** and Glycol **(E)**. (n=4, representative of 3 independent experiments with independent batches of NP, mean±SD).

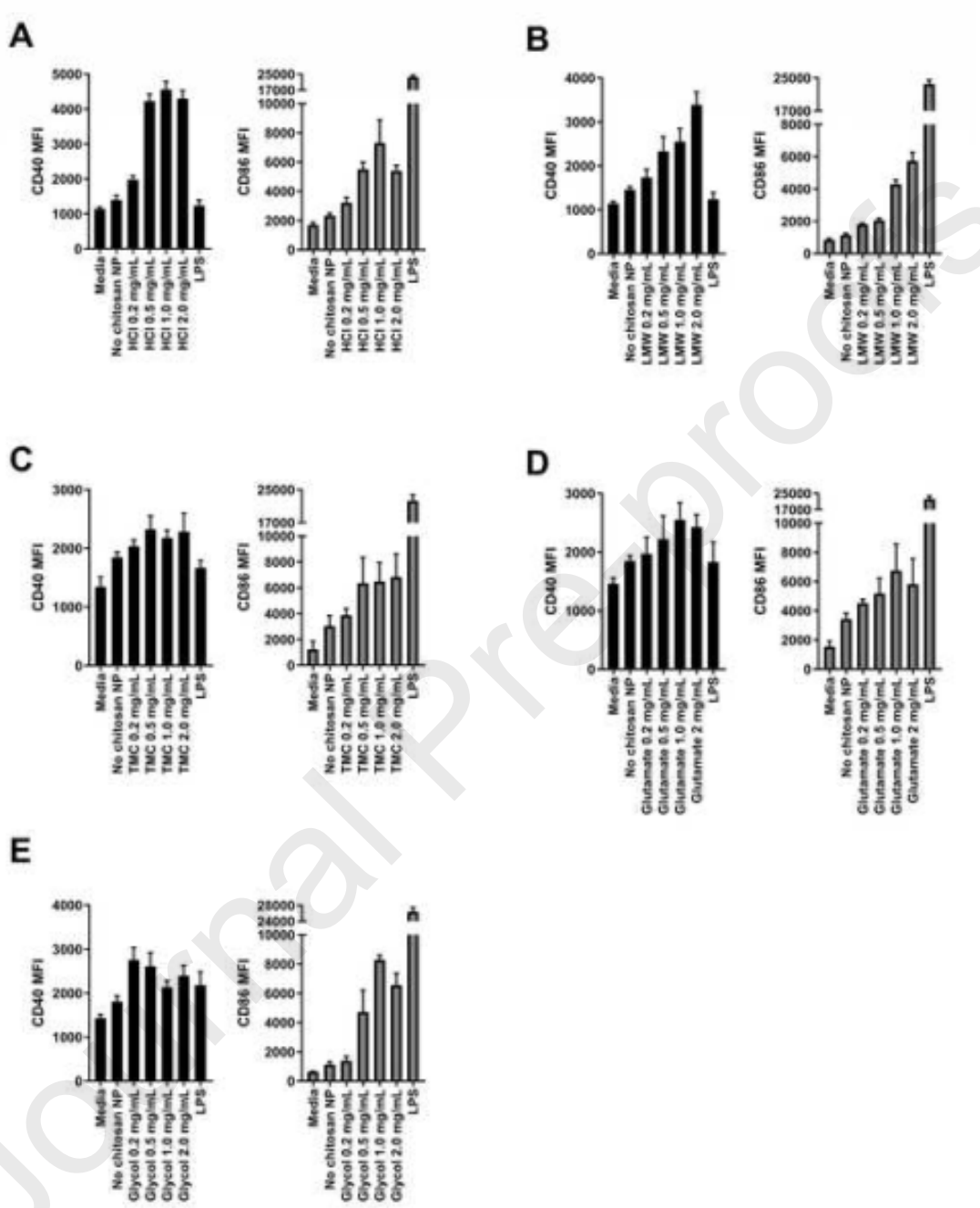
**Figure 6.** The particle size **(A)**, PDI **(B)** and zeta potential **(C)** of PLGA NPs coated with constant loading capacity of HCl, LMW, TMC Glutamate and Glycol (n=3 independent batches, mean±SD). Upregulation of CD40 **(D)** and CD86 **(E)** surface markers and cell viability **(F)** after incubation with PLGA NPs coated with chitosans for 24 h (n=4, representative of 3 independent experiments with independent batches of NP, mean±SD) (significantly higher difference compared to another group is indicated by the respective letter, $p<0.05$).

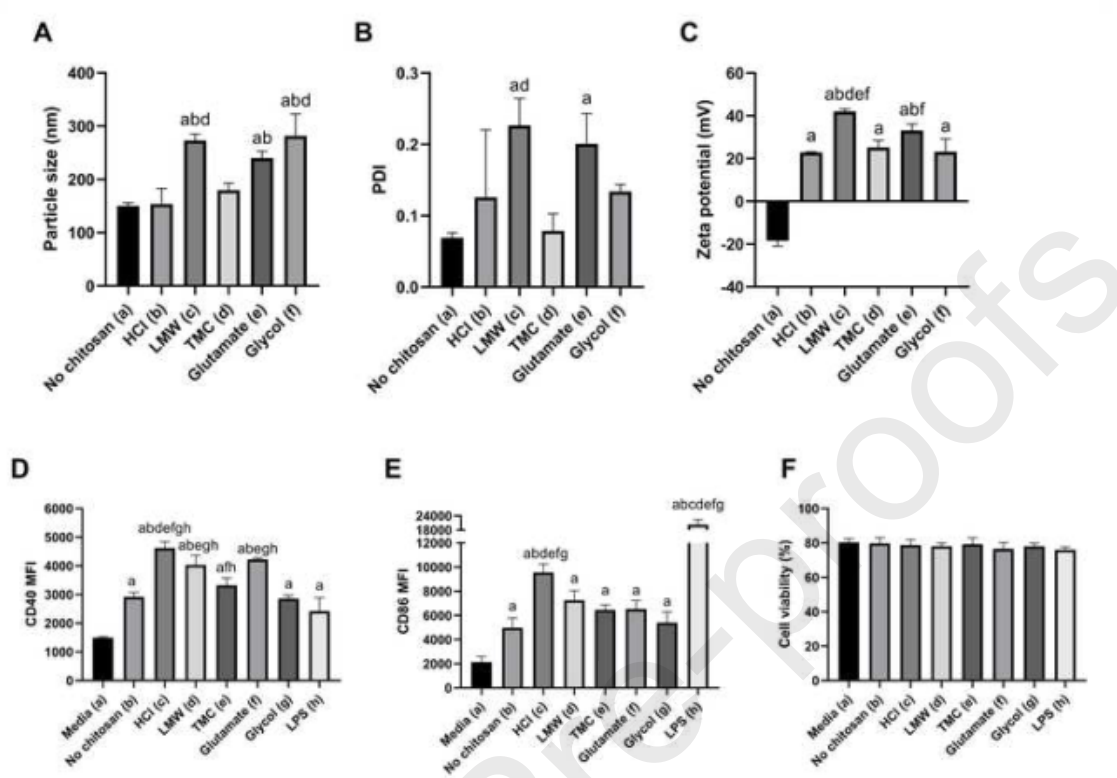












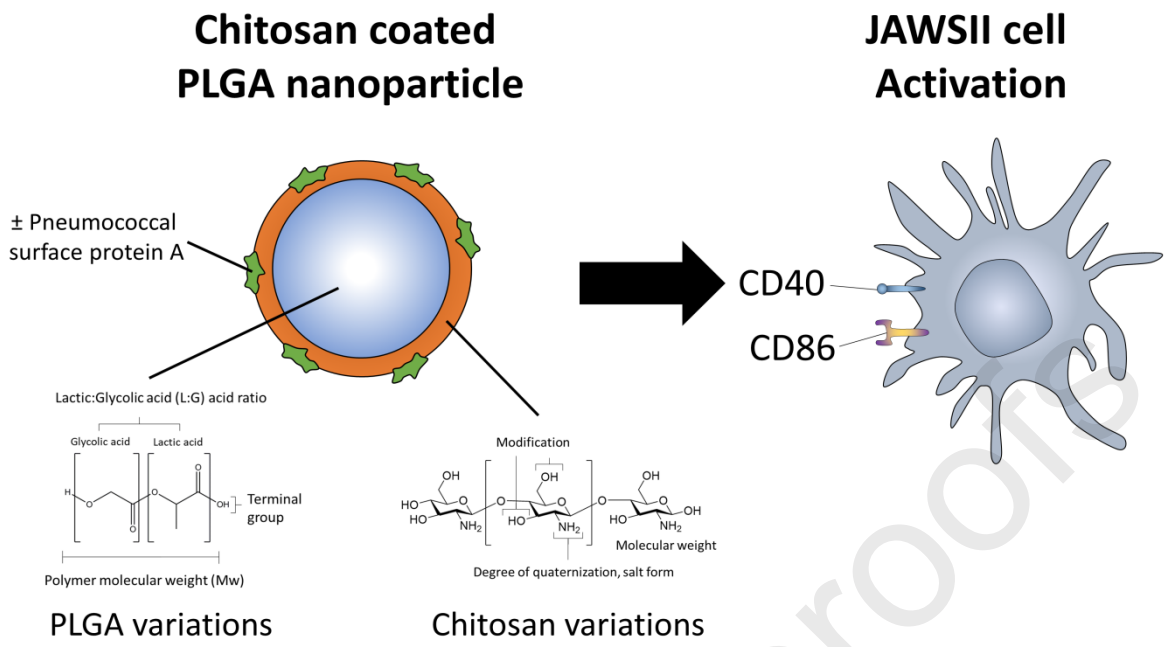


**Table 1. PLGA polymers with differing lactic acid:glycolic acid ratio, terminal group and molecular weight.**

| Polymer | L:G ratio | Terminal group | Average Mw (kDa) |
|---|---|---|---|
| REF | 50:50 | Acid | 7-17 |
| EST | 50:50 | Ester | 7-17 |
| HMW | 50:50 | Acid | 24-38 |
| LAC | 75:25 | Acid | 4-15 |

reference (REF), ester terminal group (EST), high-molecular weight (HMW) and carboxylic acid end group (LAC)

**Table 2. Different derivatives of chitosan**

| Chitosan name | Molecular weight | Degree of deacetylation | Product # |
|---|---|---|---|
| HCl | 30-400 kDa | 80-95% | 43001 HMC |
| Low molecular weight (LMW) | 50-190 kDa | 75-85% | 448869 Aldrich |
| Trimethylchitosan (TMC) | 50-190 kDa | NA | Prepared from LMW |
| Carboxymethylchitosan (CMC) | 30-500 kDa | 80-95% | 43002 HMC |
| Oligomer | <5 kDa | ≥75% | 44009 HMC |
| Glutamate | 30-200 kDa | NA | 44006 HMC |
| Glycol | 400 kDa | NA | G7753 Sigma |

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**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: